Pathways, inhibition and regulation: cell studies using MRS

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Introduction

Cancer cells harbor genetic alterations in key steps that regulate cell proliferation, differentiation, and cell-cell communication (1). These mutations endow cancer cells with the phenotypes associated with the malignant state. In their seminal review, Hanahan and Weinberg identified six essential alterations in cell physiology that collectively lead to malignant growth: self-sufficiency in growth signals, insensitivity to growth–inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (2). As our insight into the signaling pathways and regulatory networks involved in each of these alterations increases, a 'circuitry' of the mammalian cell is being elucidated (Fig.1). Furthermore, this understanding of the molecular-genetic events leading to cancer is altering therapeutic approaches. Novel rationally designed therapies are increasingly aimed at modulating the signaling pathways which are aberrant in cancer cells (3-5). However it is clear that response to such molecular targeted therapies will depend on the status of the target in the individual cancer. Thus identifying the specific mutations involved in every case is crucial to tailoring treatment and predicting response. Furthermore, in monitoring response to targeted therapies it is important to recognize that many novel treatments are associated with tumor stasis rather than shrinkage. Therefore traditional markers of response, based on tumor size, will not always be applicable. Specific molecular biomarkers are therefore needed first to identify tumors which are likely to respond to a particular molecularly targeted therapy and then to monitor response to that therapy.

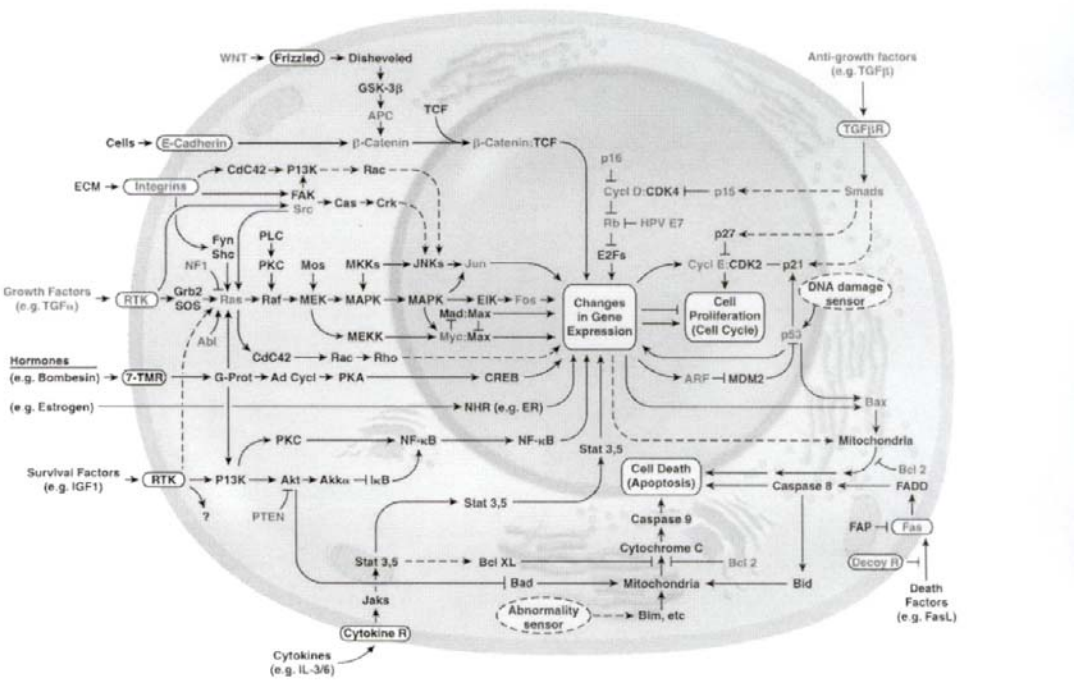

Figure 1 Integrated circuit of the cell (reproduced with permission from (2))

In this context magnetic resonance offers a non-invasive, non-destructive method that can potentially assess cell transformation and monitor response to targeted therapies. This presentation will focus on magnetic resonance spectroscopy (MRS) studies of cells and tumors. It will review investigations where MRS has been used to monitor cell transformation following specific genetic alterations as well as cellular response to therapies targeted at molecular events associated with cancer.

MRS markers of cell transformation

Increased levels of the phosphomonoesters (PME) phosphocholine (PC) and phosphoethanolamine (PE) have consistently been detected in transformed cells, tumor xenograft models and human tumors whereas response to chemotherapy and radiotherapy has been associated with a decrease in PMEs (6-9). This modulation in the levels of the cellular phospholipid precursor molecules has been typically explained as resulting from increased cell proliferation during tumorigenesis. Indeed, spheroid studies have shown that proliferating cells have a higher PC content than quiescent cells (10) and studies of an estrogen sensitive mammary model showed a correlation between the cellular S phase fraction and PC content (11). However, a comparison of human mammary epithelial cells with breast cancer cell lines, demonstrated a higher PC content in the cancer cells in spite of a comparable rate of cellular proliferation (12). Similar finding were reported in studies of a series of breast and prostate cancer cell lines where increased levels of PC were associated with cellular progression from a normal to transformed phenotype but not with the rate of cellular proliferation (13, 14).

More recently, studies have focused on linking specific molecular-genetic alterations associated with cancer with changes in the MRS spectrum of cells and tumors. The effects of immortalization and transformation of primary rat Schwann cells following expression of the SV-40 large T antigen as well as the H-ras oncogene were investigated by ^{1}H MRS. Full transformation of the cells was associated with a significant increase in PC levels as observed in the proton MRS spectrum, but no change in PC was observed following immortalization alone (15).

The effect of the tumor suppressor gene p53 was investigated using both ^{1}H and ^{31}P spectroscopy. The spectrum obtained from HCT116 colorectal cells with wild type p53 was compared to the spectrum obtained from the same cell line with a null p53 gene. A significant increase in total choline and PC content was observed following loss of p53 function (16).

The effect of constitutive over-expression of the Ras oncogene was investigated in the NIH3T3 murine fibroblast model using ^{31}P MRS. Ras overexpression resulted in an increase in PC levels by a factor of 4 as illustrated in Fig. 2. Interestingly, no increase in cellular proliferation rate was observed in the *ras* overexpressing cells. To further confirm that the increase in PC was associated with increased Ras signaling, the Ras overexpressing cells were treated with the Ras signaling inhibitors simvastatin and R115777. Inhibition of Ras signaling, as confirmed by Western blotting, was associated with a return of PC levels to those observed in control NIH3T3 cells.

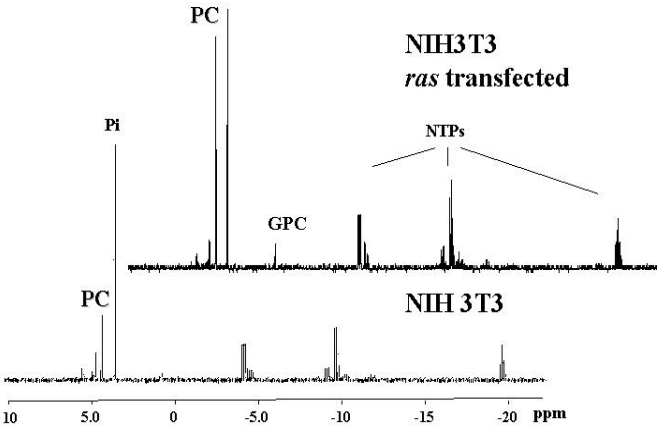

Figure 2. ^{31}P MRS spectra of NIH3T3 cells (bottom) and *ras* transfected NIH3T3 cells (top).

MRS was also used to monitor the metabolic effects of the hypoxia-inducible factor HIF-1β which is involved in inducing angiogenesis. Wild type (WT) hepatoma cells were compared to hepatoma cells deficient in HIF-1β (c4) (17). C4 tumors had a significantly lower ATP content compared to WT, while ^{31}P MRS indicated that the NTP/Pi level of the tumors was comparable. Using ^{1}H MRS the authors showed that glycine, an essential precursor for purine synthesis, was significantly lower in the c4 tumors explaining the lower ATP levels. ^{1}H MRS demonstrated that betaine, PC and choline, which are intermediates in glycine synthesis, were also lower in the c4 tumors.

MRS markers of apoptosis

As mentioned above, one of the characteristics of cancer cells is evasion of apoptosis. Re-activation of the apoptotic pathway is therefore a goal for new drug development. In addition, tumor response to traditional chemotherapeutic treatments is frequently associated with cellular apoptosis. Thus several studies have been concerned with identifying noninvasive MR markers of apoptosis (reviewed in (18-21)).

Focusing on the metabolic changes observed by MR spectroscopy, early ^{1}H MRS investigations of apoptotic leukemia cells showed that the ratio of the CH_2 to CH_3 peaks (at 1.3 ppm and 0.9 ppm respectively) was correlated with the fraction of apoptotic cells present in the sample. This was attributed to a change in membrane viscosity (22, 23). An increase in cellular lipids, as indicated by an increase in the CH_2 peak, was subsequently reported in several other cell systems, but has been associated with an accumulation of cytoplasmic lipid droplets and did not affect the CH_2/CH_3 ratio in every case (24-27). *In vivo* studies of apoptosis have also shown an increase in cellular lipids as well as an increase in polyunsaturated fatty acids (PUFA) in treated tumors (28). Interestingly a comparison of the ^{1}H spectra obtained from cells grown in culture with the same cells grown as a subcutaneous tumor indicated the increase in PUFA observed during apoptosis is observed only in the cells grown as tumors (27).

Changes have also been observed in the ^{31}P MRS spectra of cells and tumors undergoing apoptosis. An increase in CDP-choline, an intermediate in phosphatidylcholine synthesis,

was reported in some cases (29) and shown to result from inhibition of the enzyme CDP-choline:1,2-diacylglycerol choline phosphotransferase for which it is a substrate (30). A build-up in the glycolytic intermediate fructose 1,6 bisphosphate as well as glycerol 3-phosphate was observed in several cell systems following induction of apoptosis by different chemotherapeutic treatments (29, 31). This was explained as resulting from activation of the enzyme PARP following DNA damage resulting in NAD depletion and consequent inhibition of the enzyme GAPDH, downstream of fructose 1,6 bisphosphate. A recent *in vivo* study of the effects of FK866 - an inhibitor of NAD synthesis which leads to tumor apoptosis - resulted in similar MRS findings (32). A large increase was observed in the phosphomonoester peak of treated tumors, and tumor extracts confirmed the accumulation of fructose 1,6 bisphosphate and glycerol 3-phosphate in the treated tumors.

MRS markers of response to targeted therapies

Receptor tyrosine kinases (RTK) are frequently mutated or over-expressed in cancer and as such serve as targets for several novel anti-cancer treatments. ErbB2/HER2 can be inhibited with the anti-body Herceptin (trastuzumab) and its anti-tumor effects have been demonstrated in the clinic. Similarly Gleevec (STI-571) inhibits the activity of bcr/abl, c-kit and PDGFR, and is in clinical use. Finally several EGFR and VEGF inhibitors are entering the clinic (33). Downstream of RTKs several inhibitors of oncogenic signaling pathways are also being developed as potential new drugs (3-5).

Magnetic resonance spectroscopy can be used to monitor inhibition of some of these signaling pathways. Inhibition of the Ras-Raf-MEK-ERK (MAPK) pathway by the specific MEK inhibitor UO126 was investigated in a panel of breast and colorectal cell lines. In every case ^{31}P MRS showed a significant drop in PC levels which was associated with inhibition of signaling, but not always with inhibition in cell proliferation (34, 35).

Inhibition of the PI3K pathway, was also monitored by ^{31}P MRS. Using the PI3K inhibitors Wortmannin and LY294002 we have shown that inhibition of this pathway is associated with a drop in PC levels and in some cases an increase in GPC, the breakdown product of the membrane lipid phosphatidylcholine (36) possibly due to increased activity of the enzyme phospholipase A2 following down-regulation of its inhibitor annexin I (37).

MRS was also used to determine the effect of the HSP90 inhibitor 17-allyamino,17-demethoxygenldananmycin (17AAG). By inhibiting the molecular chaperone, 17AAG leads to simultaneous depletion of several known onco-proteins including c-Raf-1 (part of the MAPK pathway), AKT (part of the PI3K pathway), ErbB2/HER2, mutant p53 etc. Thus treatment with 17AAG results in inhibition of several signaling pathways associated with cell transformation and is now in clinical trials (38). ^{31}P MRS studies of colorectal and breast cell lines have shown a consistent and significant increase in PC as well as GPC following response to 17AAG treatment (39) (Fig. 3). The increase in GPC levels is probably due to inhibition of the PI3K pathway by depletion of AKT. However the increase in PC is unexpected since elevated levels of PC are typically associated with oncogenic transformation and cell proliferation rather than inhibition of oncogenic signaling pathways and cell division. Importantly, an increase in PMEs was also observed in a xenograft model following response to 17AAG (39) and MRS studies of patients participating in a phase II trial of 17AAG are currently underway (40).

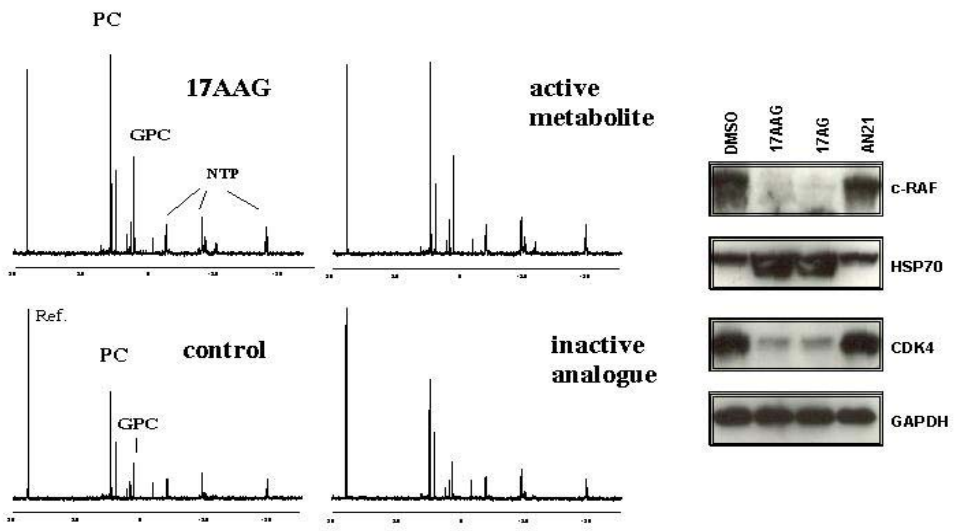

Figure 3 ^{31}P MRS spectra of HT29 colorectal cells treated with carrier (control), 17AAG, 17AG the active metabolite of 17AAG, and AN21 the inactive analogue of 17AAG. Western blots illustrate the effect of treatment on c-RAF-1, HSP70 and CDK4 proteins.

Conclusion

In summary, MRS can be used in cell and xenograft models to monitor metabolic biomarkers of the modulation of signaling pathways during oncogenic transformation or targeted treatment response. Such studies can, and in some cases have, been translated into the clinical arena. Further work is underway to develop more direct approaches for monitoring gene activation and inhibition by assessing changes in metabolite levels which are directly and specifically altered by modulation of signaling pathways during transformation and response (41).

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